Differential Down-Regulation of Protein Kinase C Subspecies in Normal Human Melanocytes: Possible Involvement of the ζ Subspecies in Growth Regulation

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Normal human melanocytes are often grown *in vitro* in the continuous presence of 12-O-tetradecanoylphorbol-13-acetate (TPA) for growth *in vitro*. The expression of protein kinase C (PKC) subspecies, which are the major cellular receptors for phorbol esters, was examined in melanocytes after long-term treatment with TPA to investigate the role of PKC subspecies in TPA-dependent cell growth. The PKC enzyme activity detected in quiescent melanocytes was almost completely depleted in cells after incubation with 85 nM TPA for 48 h. Immunoblot analysis indicated that, among the PKC subspecies α, β, δ, ε, and ζ expressed in quiescent cells, α-, β-, δ-, and ε-PKC were significantly down-regulated, whereas ζ-PKC remained at detectable levels in TPA-treated cells. TPA did not significantly affect the expression or subcellular distribution of ζ-PKC in melanocytes. Immunoprecipitation assay revealed that the enzyme activity of ζ-PKC was increased in both the cytosol and particulate cell fractions, but the increase was much greater in the latter. The activation of ζ-PKC lasted for 24 to 48 h after the addition of TPA; thereafter, ζ-PKC activity returned to basal levels. DNA synthesis was shown to change concomitantly with the activation of ζ-PKC in TPA-treated cells. These results indicate that TPA induces not only the down-regulation of α-, β-, δ-, and ε-PKC, but also long-term activation of ζ-PKC in melanocytes, and that activation of ζ-PKC parallels the growth of normal human melanocytes. *Key words: TPA/mitogenesis. J Invest Dermatol 105:567–571, 1995*

Eisinger and Marko [1] first reported in 1982 that normal human melanocytes require the continuous presence of the phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA), for their proliferation *in vitro*. It is generally accepted that the major intracellular receptor for TPA is protein kinase C (PKC) [2], a multifunctional serine/threonine protein kinase that plays crucial roles in signal transduction [3,4]. PKC is activated by increased amounts of membrane diacylglycerols that result from agonist-induced hydrolysis of inositol phospholipids. TPA activates PKC directly in a manner analogous to that of diacylglycerol both in cells and in a cell-free system. In a variety of tissues and cell types, TPA induces persistent activation of PKC and then elicits depletion of PKC or "down-regulation" [2]. Molecular cloning and biochemical analysis have revealed the existence of multiple subspecies of PKC, and more than ten subspecies of PKC, including α, βI, βII, γ, δ, ε, η, θ, ζ, and λ, have been identified in mammalian tissues [4]. Some members of the PKC family exhibit differential enzymatic properties and distinct patterns of tissue expression and intracellular localization, and thus each member of the family is suggested to play a discrete role in the processing and modulation of a variety of physiologic and pathologic responses to external signals [3,4].

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Abbreviation: MBP, myelin basic protein.

Since the demonstration that normal human melanocytes require the continuous presence of TPA for growth, much attention has been focused on the mechanism of the proliferation of melanocytes induced by TPA [5,6]. The long-term action of TPA is thought to be important for the growth of melanocytes, as membrane-permeant diacylglycerols, which are transient activators of PKC, are unable to stimulate the growth of these cells [5]. Membrane-permeant diacylglycerols are known simply to activate PKC, but do not induce PKC down-regulation. Thus, it has been postulated that TPA-dependent growth of melanocytes is a consequence of the down-regulation of PKC induced by tumor-promoting phorbol esters [5]. Overexpression of α-PKC is associated with reduced growth and increased expression of differentiation markers in melanoma cells [7]. Therefore, PKC has been proposed to inhibit the proliferation of these cells. The present study was conducted to investigate the long-term effects of TPA on each PKC subspecies. TPA treatment caused down-regulation of PKC subspecies α, β, δ, and ε but did not affect the expression of ζ-PKC in melanocytes. Furthermore, TPA induced long-term activation of ζ-PKC in melanocytes. These results suggest that PKC subspecies such as α, β, δ, and ε may be involved in the inhibition of the mitogenic response and that TPA-activated ζ-PKC might play a role in the growth regulation of normal human melanocytes.

MATERIALS AND METHODS

Cell Culture Normal human melanocytes were isolated from infantile foreskins as described [8]. Melanocytes were maintained in Ham's F-10 medium (American Biorganics, Inc., Tonawanda, NY) containing 10% fetal bovine serum (GIBCO Laboratories, Grand Island, NY), 200 U/ml peni-

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cillin, 100 μg/ml streptomycin, 85 nM TPA (Sigma Chemical Co., St. Louis, MO), and 0.1 mM isobutylmethylxanthine (Sigma) (standard growth medium).

Preparation of Total PKC Extract and Cytosol and Particulate Fractions Melanocytes were cultured in standard growth medium without TPA for 72 h to render the cells quiescent. Cells were then treated with 85 nM TPA for 48 h or for the indicated time periods. Quiescent and TPA-treated melanocytes (3–5 × 10^7 cells) were washed with phosphate-buffered saline and harvested by scraping in phosphate-buffered saline. Cells were then suspended in 4 volumes of buffer A: 20 mM Tris/HCl (pH 8.0) containing 10 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 2 mM ethylenediaminetetraacetic acid (EDTA), 10 mM 2-mercaptoethanol, 100 μg/ml leupeptin, 50 μM (p-amidinophenyl) methanesulfonyl fluoride hydrochloride (p-APMSF), and 0.5% (v/v) Triton X-100. Cells were then lysed by sonication using five 5-second bursts by a sonifier (Ultrasonic Processor Model VC50 with 2-mm probe; Sonics & Materials, Inc., Danbury, CT) and centrifuged at 100,000 × *g* for 60 min. The supernatant was used as the total PKC extract. For subcellular fractionation of melanocytes, cells were suspended in 4 volumes of buffer A without Triton X-100, lysed by sonication as described above, and centrifuged at 100,000 × *g* for 60 min. The resulting supernatant was used as the cytosol fraction. The pellet was lysed by sonication in buffer A and centrifuged at 100,000 × *g* for 60 min, and the supernatant was used as the particulate fraction.

Mono-Q Column Chromatography Equal amounts of protein (17 mg) of the total PKC extracts of quiescent and TPA-treated melanocytes were applied separately to a Mono-Q HR 10/10 column (1 × 10 cm; Pharmacia, Piscataway, NJ) connected to a fast-protein liquid chromatography system (Pharmacia) and equilibrated beforehand with buffer B: 20 mM Tris/HCl (pH 8.0) containing 0.5 mM EGTA, 0.5 mM EDTA, 20 mM 2-mercaptoethanol, 20 μg/ml leupeptin, 50 μM p-APMSF, 10% (v/v) glycerol, and 0.02% (v/v) Triton X-100. After washing the column with 10 ml of buffer B, we eluted PKC by application of a 50-ml linear concentration gradient of NaCl (0–0.5 M) in buffer B at a flow rate of 0.5 ml/min. Fractions of 1 ml each were collected. PKC activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into myelin basic protein (MBP) essentially as described [9,10]. The reaction mixture (50 μl) contained 20 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, 10 μM [γ-^{32}P]ATP, 200 μg/ml MBP, 16 μg/ml phosphatidylserine, 1.6 μg/ml diolein, 10 μM $CaCl_2$, and 5 μl of enzyme. Basal activity was measured in the presence of 0.5 mM EGTA instead of phosphatidylserine, diolein, and $CaCl_2$. After incubation for 15 min at 30°C, the reaction was terminated by spotting a 40-μl aliquot of the reaction mixture onto P81 paper (Whatman, Clifton, NJ). The paper was washed five times for 5 min each by immersion in 75 mM H_3PO_4. Radioactivity was quantitated by Cherenkov counting using a scintillation liquid spectrometer.

Immunoblot Analysis Immunoblot analysis was carried out after partial purification of PKC by diethylaminoethyl (DEAE)-cellulose column chromatography to reduce nonspecific binding. Total PKC extracts and cytosol and particulate fractions of quiescent and TPA-treated melanocytes were applied separately to a DEAE-cellulose (DE-52; Whatman) column (0.5 × 1 cm), which was equilibrated beforehand with buffer C: 20 mM Tris/HCl (pH 8.0) containing 0.5 mM EGTA, 0.5 mM EDTA, and 10 mM 2-mercaptoethanol. After the column was washed with buffer C containing 20 mM NaCl, PKC was eluted with 0.7 ml of buffer C containing 400 mM NaCl. Equal amounts of protein (33 μg/lane) eluted from the DEAE-cellulose column were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 7.5% gel and transferred to an Immobilon membrane (Millipore Corp., Bedford, MA). The membrane was incubated overnight at room temperature with Tris/NaCl buffer (10 mM Tris/HCl, pH 7.5, and 150 mM NaCl) containing 3% gelatin and 0.02% NaN_3, and then allowed to react with polyclonal antibody specific for α-, β-, δ-, ε-, and ζ-PKC (CKpV5α-a, CKpV1β-a, CKpV3δ-a, CKpV5ε-a, and CKpV5ζ-a, respectively [11]) for 1 h at room temperature. After washing with Tris/NaCl buffer containing 0.05% Tween 20, the membranes were incubated for 30 min at room temperature with the biotinylated anti-rabbit IgG antibody, and subsequently with the avidin-biotinylated horseradish peroxidase complex (Vectastain Elite; Vector Laboratories, Burlingame, CA). The color reaction was developed using diaminobenzidine tetrahydrochloride and H_2O_2. The α-, β-, δ-, and ε-PKC purified from rat brain [9,10,12] and the recombinant ζ-PKC overexpressed in Chinese hamster ovary cells [13] were used as authentic enzyme samples.

Immunoprecipitation of ζ-PKC Quiescent and TPA-treated melanocytes were washed three times with ice-cold phosphate-buffered saline and scraped into buffer D: 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 5 mM EGTA, 15 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM dithiothreitol, 20 μg/ml leupeptin, and 50 μM p-APMSF. Cells were sonicated using five 5-second bursts and centrifuged at 100,000 × *g* for 15 min. The supernatant was used as the cytosol. Pellets were lysed in buffer D containing 1% (v/v) Triton X-100, sonicated using eight 5-second bursts, and centrifuged at 100,000 × *g* for 15 min. The supernatant was used as the particulate. Equal amounts of protein (0.2 mg for the cytosol and 0.1 mg for the particulate) were immunoprecipitated with 4 μl (for the cytosol) and 2 μl (for the particulate) of anti–ζ-PKC antibody (CKpV5ζ-a) for 16 h at 0–4°C. After addition of 20 μl (for the cytosol) and 10 μl (for the particulate) of protein-A–coupled Sepharose (Pharmacia), the mixture was incubated for another 1 h with shaking. The resulting immune complex was centrifuged and washed twice with buffer D containing 0.5% Triton X-100, and once with buffer E: 20 mM HEPES (pH 7.5) containing 0.1 mM Na_3VO_4, 0.1 mM dithiothreitol, 50 μg/ml leupeptin, and 50 μM p-APMSF. The immune complex was resuspended in 20 μl of buffer E and used for kinase assay of ζ-PKC. The activity of ζ-PKC was assayed by adding 10 μl of the enzyme preparation described above to 15 μl of the reaction mixture containing 20 mM HEPES (pH 7.5), 4 mM $MgCl_2$, 10 μM [γ-^{32}P]ATP, 200 μg/ml MBP, and 30 μg/ml phosphatidylserine. After incubation for 7 min at 30°C, the reaction was stopped by addition of SDS-PAGE sample buffer, and the samples were boiled for 5 min. These samples were subjected to SDS-PAGE using a gradient (5% to 20%) gel, and the phosphorylation of MBP was visualized by autoradiography. Radioactivity incorporated into the MBP band was quantitated by measuring the intensity of photostimulated luminescence using a bioimaging analyzer (BAS 2000; Fuji Film, Tokyo, Japan).

Thymidine Incorporation Assay Quiescent melanocytes (5 × 10^5 cells in 1 ml of culture medium per well) were stimulated with 85 nM TPA. For

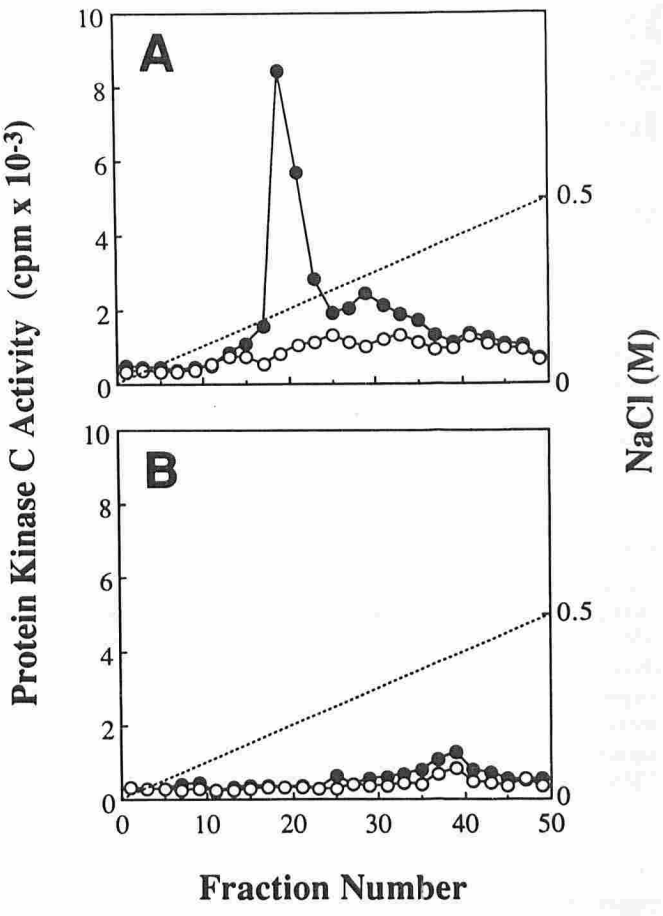


Figure 1. TPA induces depletion of PKC activity. The total PKC extracts of quiescent (*A*) and TPA-treated (*B*) melanocytes were subjected separately to Mono-Q column chromatography, and PKC activity was assayed as described in *Materials and Methods*. *Solid circles*, in the presence of phosphatidylserine, diolein, and $CaCl_2$; *open circles*, in the presence of EGTA instead of phosphatidylserine, diolein, and $CaCl_2$; *dashed line*, NaCl concentration.

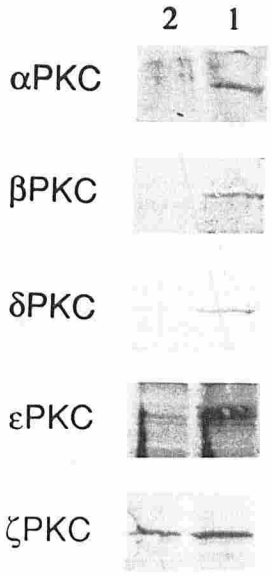


Figure 2. TPA induces differential down-regulation of PKC subspecies. The total PKC extracts of quiescent (*lane 1*) and TPA-treated (*lane 2*) melanocytes were applied on DEAE-cellulose column, and eluates were subjected to immunoblot analysis using antibodies against each PKC subspecies, as described in *Materials and Methods*. Immunoreactions of each PKC subspecies were confirmed by authentic enzyme samples.

the last 6 h of each incubation, the cells were cultured with [^{3}H]thymidine (0.5 μCi per well). Acid-precipitable materials were collected on a glass filter, and radioactivity was quantitated with a liquid scintillation spectrometer.

Protein Determination Protein content was determined by the method of Bradford [14].

RESULTS

TPA-Treated Melanocytes Show Depletion of PKC Activity To examine the long-term effects of TPA on PKC activity, we assayed PKC activities from quiescent and TPA-treated melanocytes after Mono-Q column chromatography. Distinct PKC activity was detected in quiescent melanocytes **(Fig 1*A*)**. In contrast, PKC activity was markedly reduced in TPA-treated cells, with very low activity observed in fraction numbers 29–45 **(Fig 1*B*)**.

PKC Subspecies Are Differentially Down-Regulated in TPA-Treated Melanocytes Immunoblot analysis using antibodies specific for each PKC subspecies was performed to examine the depletion of each PKC subspecies **(Fig 2)**. Types α-, β-, δ-, ϵ-, and ζ-PKC were expressed in quiescent melanocytes. Comparison

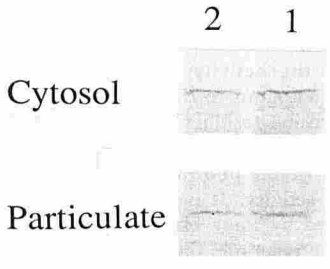
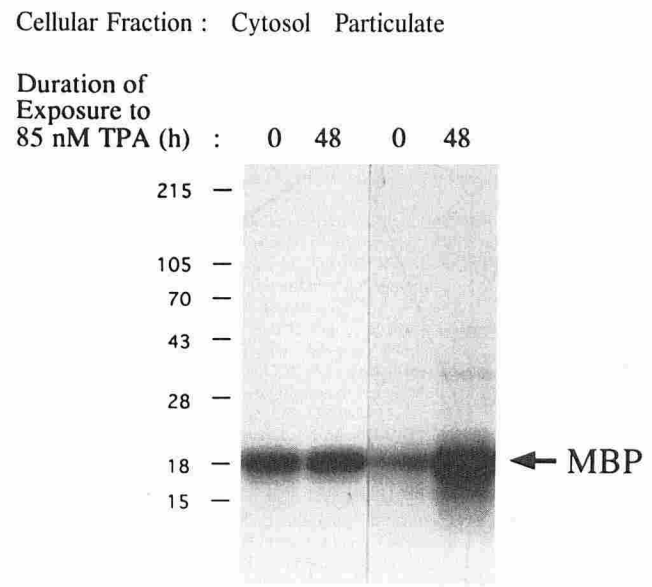


Figure 3. Subcellular distribution of ζ-PKC. The cytosol and particulate fractions of quiescent (*lane 1*) and TPA-treated (*lane 2*) melanocytes were applied on DEAE-cellulose column, and eluates were subjected to immunoblot analysis using an antibody against ζ-PKC, as described in *Materials and Methods*.



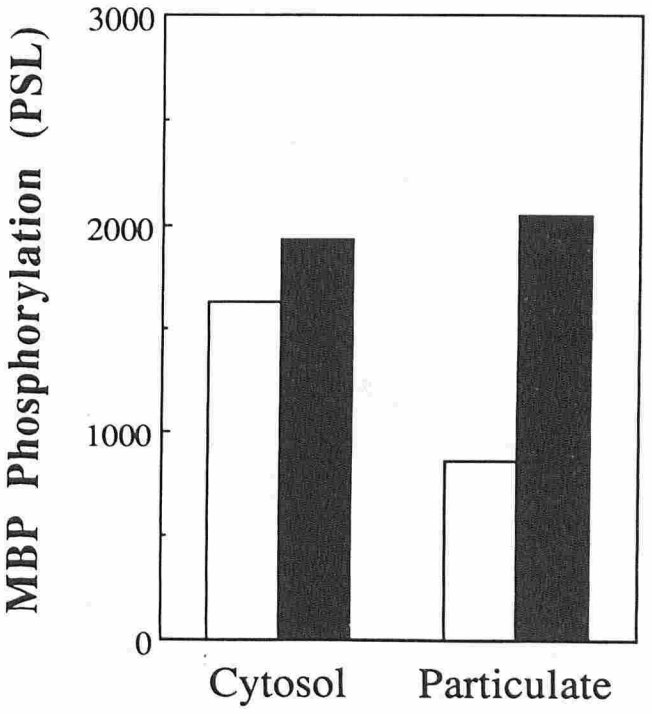


Figure 4. TPA induces activation of ζ-PKC. ζ-PKC in the cytosol and particulate from quiescent and TPA-treated melanocytes was immunoprecipitated and assayed as described in *Materials and Methods*. Phosphorylation of MBP was visualized by autoradiography (*top*) and analyzed by a bioimaging analyzer (*bottom*). PSL, photostimulated luminescence.

of immunoreactive PKC proteins in quiescent and TPA-treated cells revealed a marked decrease in α-, β-, δ-, and ϵ-PKC levels in TPA-treated cells. Both α- and β-PKC were undetectable, and δ- and ϵ-PKC were resolved only faintly in TPA-treated cells. In contrast, ζ-PKC was present at essentially the same levels in TPA-treated cells and in quiescent cells, decreasing slightly after TPA treatment. Thus, α-, β-, δ-, and ϵ-PKC proteins, but not ζ-PKC, are down-regulated in TPA-treated cells. Subsequent analysis of the subcellular localization of ζ-PKC **(Fig 3)** revealed ζ-PKC in both the cytosol and particulate fractions in quiescent melanocytes, and the immunoreactions of ζ-PKC in each fraction were not altered by

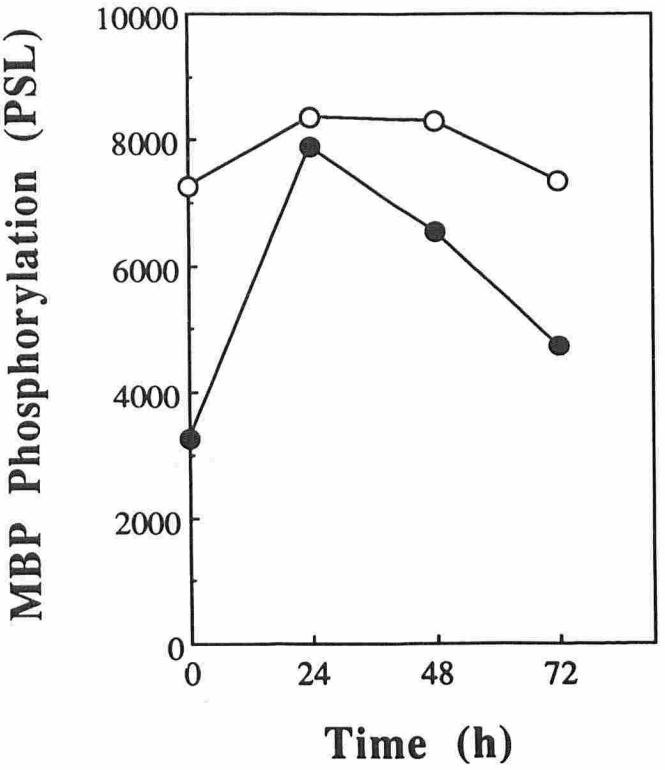


Figure 5. Time course of ζ-PKC activation by TPA treatment. Melanocytes were treated with TPA for the indicated time periods. ζ-PKC in the cytosol (*open circles*) and particulate (*solid circles*) was immunoprecipitated and assayed as described in *Materials and Methods*. Phosphorylation of MBP was analyzed by a bioimaging analyzer. Results are representative of three independent experiments. PSL, photostimulated luminescence.

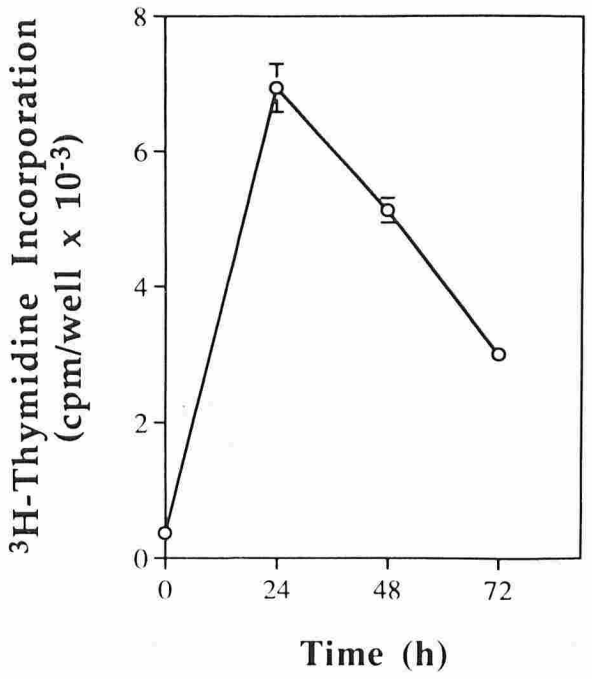


Figure 6. Time course of the effect of TPA on DNA synthesis. Quiescent melanocytes were stimulated with TPA for the indicated time periods, and incorporation of [^{3}H]thymidine into cells was measured as described in *Materials and Methods*. Data points represent the mean ± SD of triplicate cultures.

TPA treatment. These results indicate that TPA does not induce translocation or down-regulation of ζ-PKC.

ζ-PKC Is Activated in TPA-Treated Melanocytes The enzyme activity of ζ-PKC was assayed after immunoprecipitation to remove other protein kinases. Immunoprecipitated ζ-PKC was quantitated by phosphorylation of MBP in the presence of phosphatidylserine without Ca^{++} **(Fig 4)**, because ζ-PKC is active under these conditions [15–18]. TPA treatment induced activation of ζ-PKC in both the cytosol and particulate. ζ-PKC activity increased slightly in the cytosol and approximately 2.4-fold in the particulate. Time-course analysis indicated that ζ-PKC activity in the particulate was highest 24 h after TPA treatment among the time points examined and returned to basal levels at 72 h, whereas ζ-PKC activity in the cytosol increased slightly during the time course **(Fig 5)**.

ζ-PKC Activity Correlates with DNA Synthesis in Melanocytes To examine the relation between ζ-PKC activity and DNA synthesis of melanocytes, we performed a thymidine incorporation assay **(Fig 6)**. DNA synthesis was highest 24 h after the addition of TPA and decreased to basal levels in a pattern that paralleled the change in ζ-PKC activity. Thus, ζ-PKC activity, especially in the particulate fraction, changes concomitantly with DNA synthesis of melanocytes.

DISCUSSION

Normal human melanocytes are known to express several PKC subspecies. The α-, β-, and ε-PKCs have been detected by immunoblot analysis [6], and the presence of α-, β-, δ-, and ζ-PKC transcripts has been demonstrated by reverse transcriptase-polymerase chain reaction [19]. All of these PKC subspecies were detected by immunoblot analysis in this study. After TPA treatment, α-, β-, δ-, and ε-PKC are down-regulated, whereas ζ-PKC is reportedly neither translocated nor down-regulated in response to acute or chronic exposure to TPA [17,18,20–23]. In the present study, we found that α-, β-, δ-, and ε-PKC expression in melanocytes is down-regulated as in other cells. In light of the proposal that TPA-dependent growth of melanocytes is a consequence of the down-regulation of PKC [5], it seems possible that some PKC subspecies, especially α- and β-PKC, which are depleted completely by TPA treatment, may be responsible for inhibition of the mitogenic response in melanocytes.

Our results also indicate that the expression and distribution of ζ-PKC are not affected by long-term treatment with TPA in melanocytes. Because ζ-PKC has been suggested to play a crucial role in mitogenesis [24,25] and ζ-PKC is expressed exclusively in melanocytes growing *in vitro*, a role for ζ-PKC in melanocyte proliferation is possible. Previous studies have demonstrated the insensitivity of ζ-PKC to TPA in a cell-free system [15–18]. We therefore focused on the effect of TPA on ζ-PKC activity in melanocytes. TPA induced long-term activation of ζ-PKC in these cells, although the mechanism of induction remains unclear. One possibility is that the activity of ζ-PKC is regulated through phosphorylation, analogous to the reported phosphorylation of α- and δ-PKC on tyrosine residues [26–29] and the control of their activity through phosphorylation [26–30]. Some PKC subspecies activated by TPA might phosphorylate ζ-PKC, resulting in the modulation of its activity. It is also possible that the activity of ζ-PKC is controlled by some binding proteins to ζ-PKC. Several proteins such as the receptors for activated C-kinases [31], Bruton tyrosine kinase [32], and RAC protein kinase [33] have been shown to bind to PKC. These binding proteins might be phosphorylated by TPA-activated PKC, and the phosphorylated binding proteins may act to modulate ζ-PKC activity. Further studies are required to elucidate the detailed biochemical mechanism of ζ-PKC activation in melanocytes.

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